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Bioeffects of Cultured Human Cells from High Energy, Ultrashort Pulse Laser-Light

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The use of laser light for targeting devices and weapons has sharply increased the likelihood that aircrew and support personnel will be exposed to laser light during operations. The increased potential for exposure of humans highlights the fact that there is a need for scientifically based safety standards for laser exposure at the ultrashort pulse lengths. Current safety standards are largely extrapolations of exposure limits at longer pulse lengths using a minimal visible lesion endpoint in the Rhesus monkey retinal model. A non-animal model for assessing laser-light damage to tissue, particularly human, is quite desirous for obvious scientific, political, and fiduciary reasons. I assessed the sublethal insult to human cells using a tissue culture system for specific genes that have been shown to be important in several biological processes that could lead to cancer or cell death. Using the CAT-Tox (L) (Xenometrix, Inc.) assay, it appears that 532 nm nanosecond pulse of laser light are sensed and induces several stress response genes including FOS in a roughly dose dependent fashion. This approach provides insight into a more global methodology for characterizing environmental stressors via genetic profiling.

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Bioeffects of Cultured Human Cells from High Energy, Ultrashort Pulse Laser-Light Exposure

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The use of laser light for targeting devices and weapons has sharply increased the likelihood that aircrew and support personnel will be exposed to laser light during operations. The increased potential for exposure of humans highlights the need for scientifically based safety standards for laser exposure at the ultrashort pulse lengths. Current safety standards are largely extrapolations of exposure limits at longer pulse lengths using a minimal visible lesion endpoint in the Rhesus monkey retinal model. A non-animal model for assessing laser-light damage to tissue, particularly human, is quite desirous for obvious scientific, political, and fiduciary reasons. I assessed the sublethal insult to human cells using a tissue culture system for specific genes that have been shown to be important in several biological processes that could lead to cancer or cell death. Using the CAT-Tox (L) (Xenometrix, Inc.) assay, it appears that 532 nm, nanosecond pulses of laser light is sensed and induces several stress response genes, including FOS a proto-oncogene, in a roughly dose dependent fashion. This approach provides insight into a more global methodology for characterizing environmental stressors via genetic profiling.
Key Words: laser bioeffects, gene profile, laser safety

Background

The outermost layer of the retina, the Retinal Pigment Epithelium (RPE), plays a critical role in the physiology of the underlying photoreceptors[1]. Military and civilian technology of the 21st century will increasingly rely on the use of laser light, and thus increase the chances that personnel will be intentionally or accidentally optically exposed. Current safety standards for laser light exposure to the eye are based largely on whole animal minimal visible retinal lesion studies, and do not take into account the possibility of subtle sub-lethal long term effects which may become manifest long after acute treatment. Furthermore, current treatment of laser-exposed patients is concluded after visible lesions abate. And there is not a thorough scientific understanding of the laser-light damage mechanisms at the cell and molecular level. Therefore, it would of great benefit to develop, validate, and genetically engineer a tissue culture based methodology with cell lines possessing qualitatively and quantitatively sensitive damage-induced reporter gene systems.

Such cell lines would provide an *in vitro* model of the laser-tissue interaction in the eye, which could serve as the test bed for a variety of experiments leading to the development of sub-lethal laser exposure safety limits. In order to engineer a retinal pigment epithelial cell line, we must first investigate the generic genetic response, if any, of human cells to laser-light exposure. In this experiment, using a battery of 13 known damage-induced reporter genes, we assessed the genetic response of human cells grown in a tissue culture format to laser-light insult. The development of a non-animal model for assessing laser-light damage to living tissue, particularly human, is quite

desirous for obvious scientific, socio-political and fiduciary reasons and we believe that it has become technologically possible.

Materials and methods

General description:

The purpose of this study was to investigate the gene activity induced in human cells by high energy, ultrashort pulse laser-light exposure. The CAT-Tox (L) assay, developed by Xenometrix, Inc. (Boulder, CO), is designed to detect transcriptional responses to a variety of compounds including DNA damaging agents and oxidative stressors in human liver cells (Xenometrix, 1996). This gene profile assay uses a human liver cell line (HepG2) and 13 mammalian gene reporter constructs driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene. This assay was selected because it was technically appropriate, commercially available and relatively easy to adapt to laser bioeffects investigation.

In this particular series of experiments, a battery of 13 human stress response genes (briefly described in appendix 1) in reporter gene constructs known to be induced by various types of cellular stress or damage were used to assess the bioeffects of laser light exposure on human cells at the cell and molecular level. This mammalian gene profile assay is capable of measuring differential gene expression in the human hepatoma cell line, HepG2. The thirteen different recombinant human liver cell lines were generated by creating stable transfectants of different mammalian promoter-CAT gene fusions. The activity of a given promoter is quantified simply by the accumulation of CAT protein, measured using a standard CAT ELISA (Enzyme Linked Immuno-Sorbent Assay) detection system.

A broad range of promoters responsive to DNA damage, heavy metal ions, protein denaturants, aromatic hydrocarbons, retinoids, and changes in intracellular cyclic AMP levels have been included in the assay. In some cases specific response elements are monitored, permitting fine analysis of stress-regulated gene expression.

The gene profile assay [CAT-Tox (L)] has been designed to use a 96-well microtiter plate format. This system gives simultaneous dose-response information at five different exposures for the 13 recombinant cell lines. The assay yields results in 24 to 48 hours. The results are displayed in histogram form as a XenoMatrix™. The assay also includes the parental HepG2 line for the measurement of cytotoxicity. The assay can distinguish subtle differences among closely related effects, and can indicate molecular mechanisms of sub-lethal cellular injury.

Exposure:

For the CAT-Tox (L) assays the cells were divided into the following groups. Two rows of cells per 96-well plate were controls (non-lased cells). The remainder of the rows were exposed to various energies of laser-light using the 532 nm wavelength of the Nd-YAG laser (Coherent, Infinity) pulsing at 2.5 Hz at a pulse width of 3 nanoseconds (ns). The beam was shaped to as uniformly as possible fill the bottom of the well. The lowest exposure was one pulse delivering

310 mJ at the surface of the tissue culture media in the well. The highest dosage was 2 seconds (s) at 310 mJ per pulse at 2.5 Hz yielding 1550 mJ on target. Three of the exposures were assayed at 24 hours and two at 48 hours post- exposure. All pulses were delivered to the surface of 50 microliters of growth media in a 6 mm well in 96-well polystyrene (Falcon, 3072) plates containing a confluent monolayer of cells on the bottom of the well. The chart immediately below indicates the exposure and assay regimen for the data displayed in Figure 1.

Treatment	Exposure Duration (s)	mJ per Pulse	Total Incident Energy (mJ)	Post Exposure Assay Time (hr)
A (control)	0	0	0	24/48
B	10	55	1375	24
C	0.4	310	310	24
D	2	310	1550	24
E	2	284	1420	48
F	10	55	1375	48

Gene profile assay:

We adapted the manufacturer's protocol to assess the effects of laser-light exposure and they were done in triplicate. The assay involved 13 stably transfected human liver cell lines, each containing a unique stress-responsive promoter or response element fused to the Chloramphenicol Acetyl Transferase (CAT) reporter gene. In each genetically induced cell line, the CAT reporter gene was transcribed and subsequently translated. CAT production is detected by an ELISA (Enzyme Linked Immuno-Sorbent Assay) methodology yielding a quantitative measure of the stress gene induction expressed as fold induction compared to the control. Those cell lines without CAT protein production will not show gene induction above the control (non-lased cells). One Gene Profile Assay was performed, using a human liver cell line (HepG2), and 13 mammalian gene reporter constructs driving expression of the CAT gene. The assay was performed with concurrent negative controls, and assayed separately with known positive control exposures. The assays performed within acceptable limits for both positive and negative controls.

Assay procedure: A shortened version of the assay protocol is as follows.

1. The 13 recombinant cell lines and the parental HepG2 cell line are plated, one row each, over two 96-well microtiter plates.
2. The cell lines are dosed at five exposures and incubated at 37°C, 5% CO_2 for 24 or 48 hrs.
3. After the post-exposure incubation period the cells are washed two times and lysed with a detergent based buffer to release total cellular protein.
4. An aliquot of the total protein is transferred to 96-well microtiter plates containing Bradford protein dye. Incubation of the protein with the protein dye creates a color change that can be measured at optical density $(OD)_{600}$. This reading serves as a normalization factor for total cellular protein from well to well in the assay.
5. The remaining cellular protein is transferred to 96-well plates containing polyclonal anti-CAT antibodies. A standard sandwich ELISA is performed and in the final step horseradish peroxidase catalyzes a color change reaction that can be measured at OD_{405}.

6. The parental HepG2 cell line that was dosed in the same manner as the 13 recombinant lines is used to perform an MTT-based cellular viability assay. The results of this assay can be monitored at OD_{550}.
7. Xenometrix software uses the OD_{600} and OD_{405} readings to calculate the transcriptional fold induction for each recombinant cell line at each test exposure. The software also converts the OD_{550} to cellular viability percentages.

All plates containing the control and experimental cells were measured in an automated microplate reader (Bio-Kinetics, EL312e) which read their optical density (endpoint of ELISA) as a measure of CAT production or gene expression. This data was then electronically transferred to a computer database and eventually tabulated in graphic form. Normally three trials were conducted and the results were averaged (graphically represented in Figure 1). The assay allowed us to gather qualitative (which genes were activated) and quantitative (fold induction) data.

Data analysis

Cell viability:

Cell viability is calculated as the percent of the cells surviving at the assay time in the dosed samples versus the no dose control. In the gene profile assay, viabilities are recorded based on the results of MTT viability assays performed concurrent with the assay.

Gene expression calculations:

Gene expression is measured as the fold induction. That is the fold (multiple) increase of a construct at concentration *n* is determined by dividing the construct activity at each dose, *n*, by the activity at the zero dose. The levels of gene induction are expressed as multiples of basal or background values (zero dose). In the gene profile assay, background activity values are normalized and represented as 1.0 fold induction.

Results

Cell cytotoxicity:

The cell viability portion of the assayed shown along the left wall on the graph on Figure 1 indicates that the parent cell line withstood pulses of 532 nm laser-light with at or greater than 100% viability for exposures B and C. But viability decreased markedly in exposure treatments D, E and F (70-75%) as shown graphically on the left wall of Figure 1.

Human stress gene promoter induction:

The controls in this experiment were the identical cell lines that were not exposed to the laser light beam. Appropriately, these lines did not show fold induction in the assay as seen in Figure 1, and are used as a baseline for the treated cells. One of the stress reporter-gene cell lines, FOS, in this experiment showed a roughly dose-dependent induction, while three others, (NFkBRE, HSP70 and GADD153) showed more irregular weak responses. The remaining constructs did not show any marked stress response. It was noted that HSP70 showed a slight induction at the highest energy levels of exposure in the 24-hr assays, but not in the 48-hr assays. NFkBRE and GADD153 appeared at low levels only in the 48 hr assays.

Data interpretation

The results strongly indicate several important findings. First that human cells, liver in this case, have the ability to "sense" ultrashort pulse high-energy laser light exposure. Secondly, human cells can respond to sublethal laser light insult using, at a minimum, some of the same stress response genes previously characterized in response to other cellular stressors. The nearly dose-dependent response exhibited in the FOS constructs (approximately 3 fold inductions) is a particularly significant result. The FOS construct activation indicates possible DNA damage, which could have profound implications to the safety standards for laser exposure, since this implies subtle long-term effects such as carcinogenesis as FOS is a proto-oncogene. The NFkBRE has also been shown to be involved in the apoptosis regulatory pathway. Apoptosis is defined as programmed cell death or cell suicide. This finding is concordant with and possibly explains the "biological magnification" of retinal lesions non-existent at 1 hr post-exposure but present at 24 hours post-exposure. DNA damage is also a requisite for the induction of the GADD genes which are almost universally involved in repairing DNA damage. These results are also consistent with the findings of Leavitt, et.al. Low level RARE construct induction could signal DNA mutagenesis or other forms of aberrant gene activation. Retinoids are involved in numerous developmental pathways in humans. The HSP70 gene induction indicates possible heat shock and potential intracellular protein denaturation. Since the HSP70 gene was induced in the 24-hr assays and not in the 48-hour assays, it appears the heat shock was slight and easily remediated by cellular repair mechanisms. It also indicates that HSP70 is an early indicator of laser insult versus the DNA damage genes that appear to be induced within 48 hours post-exposure.

Conclusion

These results will point us in the proper direction for development of an immortalized RPE cell line genetically engineered with appropriate CAT-producing stress reporter-gene constructs. At this juncture we can say that any *in vitro* laser tissue damage assessment system should include DNA damage constructs. Cell damage repair genes such as those coded for by GADD153, HSP70 and the proto-oncogene, FOS, should also be considered for incorporation. Such constructs will provide a qualitative basis as well as a quantitative measure for damage in the retinal cells, so that more sophisticated understanding of laser induced damage at the cell and molecular level can be elucidated.

From a more global perspective, these findings provide a "proof of concept" that gene profiling technologies can be extremely efficacious in approaching the problem of laser-tissue interaction from the biological perspective. Gene profiling can provide insight into the type and degree of laser induced damage at the cell and molecular level as well as the damage thresholds for induction of repair metabolism or promotion of apoptosis or necrosis. The medical ramifications of these technologies in the context of prophylaxis and treatment are obvious.

Appendix 1: Gene Profile Assay - Liver: Promoter/Response Element Reporter Gene Constructs

1. CYPIA1. Cytochrome P450 IA1 promoter. The CYPIA1 promoter is involved in the oxidative metabolism of many exogenous chemicals and drugs[11]. Induction of this gene is controlled by the xenobiotic response (XRE) element. Inducing agents bind to the cytoplasmic Ah (aryl hydrocarbon) receptor, which is present not only in the liver, but also in other extrahepatic tissues such as lung, skin, and kidney. The Ah receptor-ligand complex translocates to the nucleus and induces transcription by binding to the XREs in the CYPIA1 gene. This gene responds to polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*] pyrene (B[*a*]P), 7-ethoxycoumarin, and 3-methylcholanthrene (3-MC). The CYPIA1 promoter can also respond to other chemicals such as b-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-diozin (TCDD), and polychlorinated biphenyls (PCBs).

2. GST Ya. Glutathione *S*-transferase *Ya* subunit promoter. The GSTs are a family of enzymes that add a thiol of reduced glutathione to a variety of electrophiles. GSTs can also directly bind to other compounds including heme, bilirubin, polycyclic aromatic hydrocarbons (PAHs), and dexamethasone. The active loci of the GSTs contains two binding sites, one site for glutathione (G-site), and a substrate binding (H-site) site[15]. GST Ya inducible transcription is controlled by two distinct response elements, the xenobiotic response element (XRE) and the antioxidate-responsive element (ARE)[15]. The XRE is identical to and transcriptionally activates GST Ya in a similar fashion as the XRE in the CYPIA1 gene. The ARE can work in an Ah (aryl hydrocarbon) receptor dependent or independent manner[15]. The ARE contributes to induction of GST Ya mediated by PAHs through interaction with the Ah receptor. Compounds that do not require the Ah receptor also activate the ARE. These compounds include the phenolic antioxidants *tert*-butylhydroquinone and 3,5-di-*tert*-butylcatechol.

3. XRE. Xenobiotic response element. Two copies of the XRE from the CYPIA1 gene have been placed upstream of the minimal thymidine kinase (TK) promoter. The arrangement allows segregation of multiple responses through the CYPIA1 and/or GST Ya promoters to isolate mechanistic differences. Inducing agents bind to the cytoplasmic Ah (aryl hydrocarbon) receptor, which is present not only in the liver but also in other extra-hepatic tissues (e.g., lung, skin and kidney). The Ah receptor-ligand complex translocates to the nucleus and induces transcription by binding to the XREs in this construct. This promoter fusion responds specifically to polycyclic aromatic hydrocarbons (PAHs) such as benxo[*a*]pyrene (B[*a*]P), 7-ethoxycoumarin, and 3-methylcholanthrene (3-MC). XRE can also respond to other chemicals such as b-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs).

4. HMTIIA. Metallothionein-II_A promoter. Metallothioneins (MTs) are a family of low-molecular weight heavy metal binding proteins. MT-IIA is classically induced by a variety of heavy metals including cadmium, zinc, copper, and silver[16] through the metal responsive element (MRE). In mammals MT-II_A is also induced by glucocorticoids. The precise functions of MTs are not known but it is hypothesized that they may act as intracellular storage sites for zinc and as sequestration sites for cadmium[9]. MTs do mediate resistance to the toxic effects of metals, and they may also play a role in cellular resistance to alkylating agents and ionizing radiation. The alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), as well as heavy metals, can transcriptionally induce MT-IIA.

5. FOS. *c-fos* promoter. *c-fos* is one of many nuclear proto-oncogenes. It may form an integral part of the AP-1 transcriptional complex along with the nuclear proto-oncogene, *c-jun*[6]. The AP-1 complex in turn binds to the TPA (12-*O*-tetradecanoylphorbol 13-acetate) response element (TRE). The TRE is present in several genes including collagenase, MT-IIA, stromelysin, interleukin-2, and *c-fos*. TPA, a phorbol ester tumor promoter, is a potent activator of the protein kinase C pathway. Other agents which lead to the activation of protein kinase C, such as serum and growth factors, can also upregulate these genes through the TRE. The *c-fos* promoter is regulated by a variety of response elements including the TRE, serum response element (SRE), and a cAMP response (CRE) element[8]. The *c-fos* has also been implicated in the mammalian UV response pathway which appears to have an important role in the response to DNA damaging agents such as methyl methanesulfonate (MMS) and 4-nitroquinoline N-oxide (4-NQO)[14].

6. NFκBRE. Nuclear factor of k enhancer (B site) response element. NFkB is a multi-subunit, transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune, and acute phase response[7]. The protein is constitutively regulated in B cells but is inducible in many other tissue types. When exposed to activating agents, NFkB is released from its inhibitor IkB in the cytoplasm, translocates to the nucleus, and then binds the NFkB RE present in several different genes. For this construct, one NFkB RE has been placed upstream of the SV40 minimal promoter. Many different agents can activate the DNA binding activity of NFkB including viruses, bacterial lipopolysaccharide, protein synthesis inhibitors, cytokines (TNF-a, TNF-b and interleukin-1), phorbol esters, lectins, and calcium ionophores[17]. NFkB is also hypothesized to be part of the UV response pathway and can therefore respond to certain classes of DNA damage agents as well[6].

7. HSP70. 70 kd heat shock protein promoter. Cells react to hyperthermia and other protein-damaging conditions by increasing the synthesis of a group of stress induced or heat shock proteins[12]. Under physiologic conditions, these proteins play an essential role in protein-protein interactions. Members of the HSP70 family participate in the folding, assembly, and translocation of intracellular proteins. HSP70 also binds to proteins unfolded as a consequence of metabolic or exogenous stress. The inducible activity of the HSP70 gene is regulated by the heat shock element (HSE) located in the promoter[18] which binds the cloned heat shock factor (HSF). This element appears to be sufficient for all of HSP70's inducible responses. The HSP70 gene can respond to a variety of agents and conditions which affect protein conformation. These include heat, cadmium, amino acids analogs, reduced pH, nonionic detergents, urea, and alkylating agents[19].

8. CRE. cyclic adenosine monophosphate (cAMP) response element. There are two major signaling pathways that utilize cAMP[8]. One involves protein kinase C and ultimately controls genes that contain a TRE (12-*O*-tetradecanoylphorbol 13-acetate response element). The other involves protein kinase A and alters gene activation through the CRE. Up-regulation of the cAMP pathway indicates that a compound activates cellular signaling specifically through protein kinases. Kinases are enzymes that catalyze the transfer of phosphate groups from ATP, ADP or AMP to a substrate. The fusion construct used in the CAT-Tox Assay contains two copies of the consensus CRE upstream of the SV40 minimal promoter. This construct responds to any agent that increases the level of cAMP in cells.

9. p53RE. p53 response element. The p53 wild-type protein is involved in transcriptional activation of genes that negatively control growth and/or invasion through the p53RE[21]. Mutant forms of p53 have been implicated in deregulation of gene expression in many tumor types. The p53RE construct contains 10 copies of the p53RE from the GADD45 gene[20] fused to the minimal thymidine kinase promoter. The p53 response element construct can be upregulated by DNA damaging agents. It is hypothesized that this may be due to p53's role in the inhibition of replicative DNA synthesis. Growth arresting agents can also induce the p53 response element construct.

10. RARE. Retinoic acid response element. Retinoids have a broad range of normal biological activities in growth and differentiation[5]. Retinoids have also been implicated in disturbing fetal development through aberrant gene activation. Retinoic acid analogs interact differentially with the retinoic acid receptors (a, b, and g) and the retinoid X receptors (a, b, and g) which can then bind to RAREs in downstream genes[13]. The RARE construct in the CAT-Tox Assay contains 2 copies of the RARE from the retinoic acid b receptor fused to the minimal thymidine kinase promoter. This construct responds to retinoic acid analogs including all-*trans*-retinoic acid and 9-*cis*-retinoid acid.

11. GADD153. 153 kd growth arrest and DNA damage promoter. GADD153 is a CCAAT/enhancer binding protein-related gene whose expression is regulated by growth arrest and DNA damage[10]. Although the GADD153 promoter has been cloned, the specific sequences responsible for the DNA damage response have not been well characterized. GADD153 has been shown to respond to a variety of DNA damaging agents including UV-mimetic, DNA-cross-linking, and alkylating agents[10]. This response appears to be directly related to altered DNA structure.

12. GADD45. 45 kd growth arrest and DNA damage promoter. The GADD45 gene was cloned at the same time as GADD153[10] and these genes were originally thought to be coordinately regulated. More recently, subtle differences in their regulation have been noted[20] including the finding that the GADD45 gene contains a p53 response element in its third intron. The GADD45 promoter has been analyzed and it has been determined that a large stretch of promoter is necessary for the DNA damage response[14].

13. GRP78. 78 kd glucose regulated protein promoter. GRP78 is a major endoplasmic reticulum (ER) protein that functions as a chaperone. It associates with a variety of other proteins in the ER that are transmembrane targeted and may also be involved in the assembly of oligomeric proteins[22]. Proteins that are malfolded because of mutagenesis, under-glycosylation, or other stress conditions in the ER associate with and upregulate the expression of GRP78. GRP78 appears to be the ER counterpart of HSP70, which resides in the cytoplasm. Depleted intracellular calcium stores and alkylating agents can also induce GRP78.

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GENE PROFILE

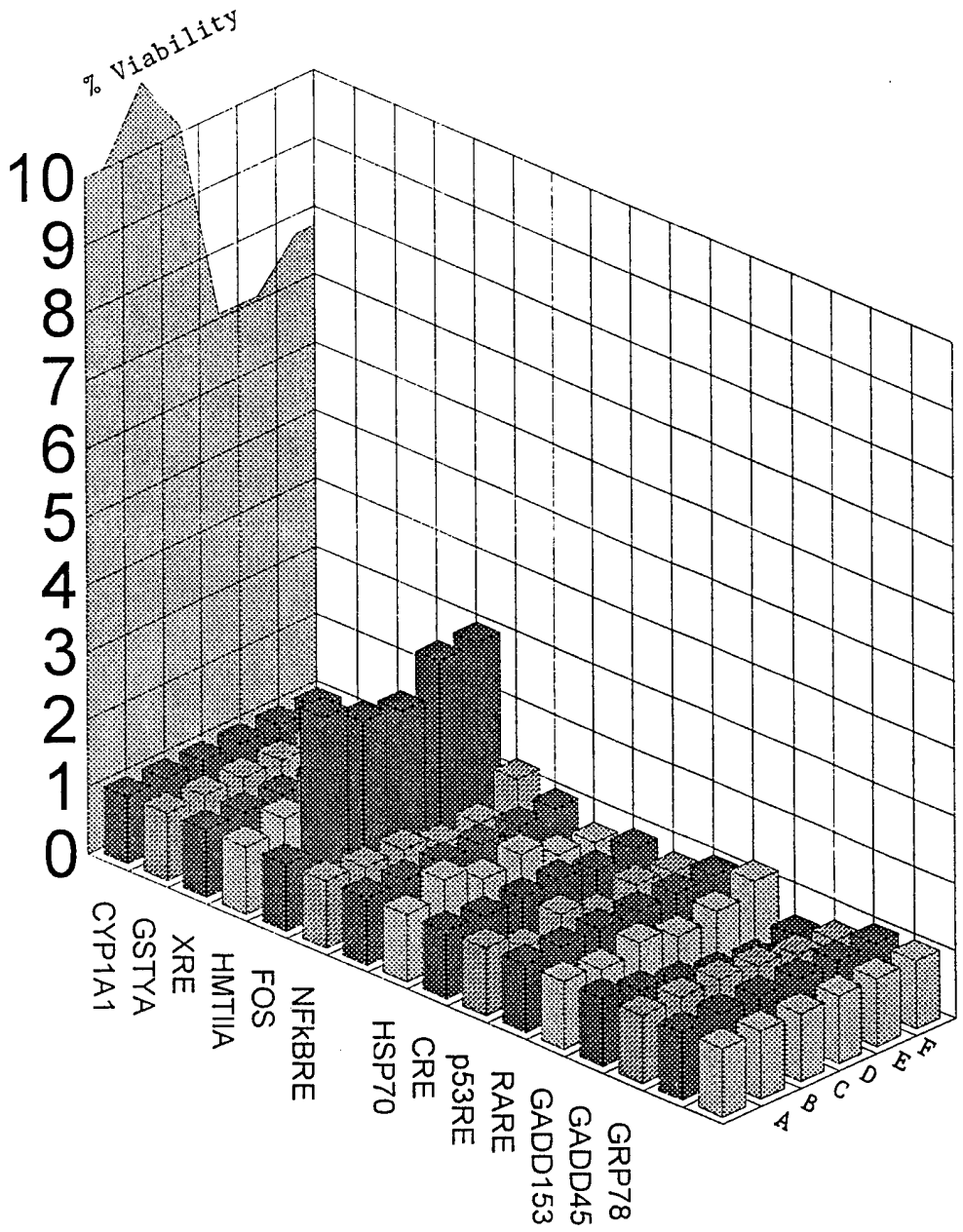

Figure 1: Fold Induction of 13 Human Stress Response genes after laser-light exposure. The vertical scale represents fold induction of the genes designated in the foreground and percent survival the parental cell line as shown on the left wall. The exposure treatments are along the right floor (A-F) and described in the text.